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THE ERYTHROCYTE Na^+/H^+ EXCHANGERS OF EEL (*ANGUILLA ANGUILLA*) AND RAINBOW TROUT (*ONCORHYNCHUS MYKISS*): A COMPARATIVE STUDY

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Summary

Trout and eel red blood cell Na^+/H^+ exchangers show widely different regulatory properties. Catecholamines, cyclic AMP and phorbol esters, which activate the trout red cell antiporter, do not affect the eel exchanger. Unlike the trout red cell exchanger, the eel red cell exchanger is strongly activated by cell shrinkage, allowing a remarkable cell volume recovery. These different regulatory properties probably indicate the existence of different isoforms of the exchangers in nucleated erythrocytes, since sensitivity to catecholamines is known to be dependent upon the presence of protein kinase A consensus sites on the cytoplasmic domain of the antiporter.

After shrinkage of eel erythrocytes, the Na^+/H^+ exchange rate gradually increases to reach a maximum value after about 10 min. The magnitude of activation is a graded function of cell shrinkage. Deactivation, like activation, is induced by a volume change and occurs after some delay (lag time).

The response of the trout antiporter (βNHE) to cell shrinkage is much reduced compared with that of the eel antiporter. In addition, the antiporter is deactivated prior to restoration of the normal control volume, leaving cell volume regulation notably defective. The trout red cell antiporter, which is desensitized and enters a refractory state following hormonal activation, is only deactivated (it can be reversibly reactivated) after shrinkage-induced activation. This dual control may occur by both phosphorylation-dependent and phosphorylation-independent mechanisms.

In view of the similarities in the regulatory properties of eel and salamander (*Amphiuma* sp.) Na^+/H^+ exchangers, the expression of a putative K^+/H^+ exchange mediated by the Na^+/H^+ exchanger was sought in eel erythrocytes. However, neither osmotic swelling nor calyculin-A-dependent phosphorylation revealed such a K^+/H^+ exchange.

Key words: Na^+/H^+ exchange, K^+/H^+ exchange, cell volume regulation, phosphorylation, erythrocyte, desensitization, eel, trout, *Anguilla anguilla*, *Oncorhynchus mykiss.*

Introduction

The amiloride-sensitive Na^+/H^+ exchanger is a plasma membrane transport protein that is activated by a number of external signals (osmotic pressure, the presence of spermatozoa, phorbol esters, growth factors, hormones and neurotransmitters) and, through such activation, plays a major role in such processes as cell volume regulation, intracellular pH homeostasis, mitogenesis and secretion. The molecular processes involved in activation and regulation of Na^+/H^+ antiporters are still not well understood (Tse *et al.* 1993). Nucleated erythrocytes obtained from non-mammalian species represent a convenient model to study the regulation of such a membrane transport protein because of their easy availability and capacity to respond to a wide range of physiological stimuli, including hormones. Moreover, acquiring mRNA from circulating erythrocytes allows the transport proteins specifically expressed in these cells to be cloned, thus enabling structure–function analyses to be carried out.

The data on Na^+/H^+ exchangers, obtained from the only two types of nucleated erythrocytes extensively studied so far, show that the regulatory properties of these exchangers are very different. In the salamander (*Amphiuma* sp.) red blood cell, Na^+/H^+ exchange has been found to be strongly activated by cell shrinkage and intracellular acidification but not by catecholamines and other hormones (Cala, 1983; Cala and

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Maldonado, 1994). Moreover, in response to swelling, a K^+_{int}/H^+_{ext} exchange is activated, allowing cell volume regulation. It is the only example, to our knowledge, involving activation of K^+/H^+ exchanges during a regulatory volume decrease and it has been suggested that these exchanges are mediated by the Na^+/H^+ antiporter while it is functioning in a different mode (Cala, 1986). In contrast, in the fish (trout) erythrocyte, no evidence of a K^+/H^+ exchange mediated by the Na^+/H^+ antiporter has been obtained (F. Borgese, F. Garcia-Romeu, H. Guizouarn and R. Motais, unpublished results). The exchange has been shown to be insensitive to intracellular acidification, poorly activated by cell shrinkage but strongly activated by catecholamines (Baroin *et al.* 1984; Nikinmaa and Huestis, 1984; Cossins and Richardson, 1985; Borgese *et al.* 1986), by cyclic AMP (Mahé *et al.* 1985) and, to a lesser extent, by phorbol esters (Motais *et al.* 1992; Guizouarn *et al.* 1993). In addition, after activation by hormones or cyclic AMP, the antiporter rapidly desensitizes and remains for hours in a refractory state (Garcia-Romeu *et al.* 1988; Guizouarn *et al.* 1993), a regulatory process not so far described in other Na^+/H^+ antiporters. The activation of the red cell antiporters in trout is controlled by the level of blood oxygen (Motais *et al.* 1987; Claireaux *et al.* 1988; Fiévet and Motais, 1991), the role of the antiporter being to increase the oxygen-transporting capacity of erythrocytes in hypoxic conditions (Nikinmaa, 1982; Nikinmaa and Huestis, 1984; Cossins and Richardson, 1985; Boutilier *et al.* 1986; Claireaux *et al.* 1988).

The trout red cell antiporter, termed βNHE because it is activated by β-agonists, has been cloned. This isoform has two cyclic-AMP-dependent protein kinase consensus sequences in the cytoplasmic C terminus (Borgese *et al.* 1992, 1994). Cloning of the *Amphiuma* isoform, which is very volume-sensitive, or that of another species with the same characteristics, would provide an insight into the mechanisms involved in activation by cell shrinkage and would also permit an evaluation of the putative ability of this Na^+/H^+ antiporter to perform K^+/H^+ exchange.

With the aim of increasing our knowledge of the divergent regulatory patterns of nucleated red cell Na^+/H^+ antiporters, we made parallel analyses of the capacities of eel and trout red cell antiporters to be activated by various stimuli and of the characteristics of their deactivation and desensitization. These results were then compared with those obtained from the *Amphiuma* antiporter by Cala.

Materials and methods

Preparation of cells

Trout [*Oncorhynchus mykiss* (Walbaum)=*Salmo gairdneri* L.] weighing approximately 250 g, obtained from a commercial hatchery and kept for 1 week in the laboratory, were stunned by a sharp blow on the head, and blood was removed from the caudal vein into a heparinized syringe. Eels (*Anguilla anguilla* L.), also weighing approximately 250 g, were obtained from the Société Piscicole de la Crau, 13280 Raphèle-les-Arles, France. The blood cells were washed in their respective saline solutions and the buffy coat removed by suction. They were then resuspended at a haematocrit of 15 % and incubated overnight at 4 °C in their saline solutions to ensure that they reached a steady state with respect to ion and water contents before experimental treatment.

Solutions

For trout red cells, the isotonic saline solution (330 mosmol l^{-1}; pH 7.55) contained (mmol l^{-1}): 145 NaCl, 5 $CaCl_2$, 1 $MgSO_4$, 4 KCl, 15 Hepes, 5 glucose. For eel red cells, the isotonic saline solution (220 mosmol l^{-1}; pH 7.55) contained (mmol l^{-1}): 85 NaCl, 5 $CaCl_2$, 1 $MgSO_4$, 4 KCl, 15 Hepes and 5 glucose.

Na^+-free solutions were obtained by substitution of NaCl by choline chloride. The osmolarity of the experimental medium was changed by varying [NaCl] or [choline chloride] or by the addition of sucrose as indicated in the Results section. All media contained 1 mmol l^{-1} ouabain.

Measurements of unidirectional sodium influx (J_{in}^{Na}), ion and water contents

The techniques used for measuring intracellular ion and water content, rate of unidirectional ^{22}Na influx (J_{in}^{Na}) and intracellular pH have been described in various publications from our laboratory (Borgese *et al.* 1986, 1987). Briefly, the cells were separated from the solution by centrifugation in nylon tubes which were then cut to liberate the pellet and the supernatant, both of which were saved for analysis. The mass of the cell pellet (wet and dry) was determined gravimetrically and expressed in g water g^{-1} dry cell solids (d.c.s.). Cell water, ion content and ^{22}Na uptake were calculated after correction for trapped extracellular fluid. The ion content was expressed in μmol g^{-1} d.c.s. All experiments were performed at 15 °C.

In order to measure unidirectional Na^+ influx, cells were suspended in the experimental saline and at regular time intervals three subsamples of this suspension were taken and incubated for 3 min with ^{22}Na (7.4×10^5 Bq ml^{-1}). The samples were centrifuged in small nylon tubes and the pellets separated by cutting the tubes. The wet mass, dry mass and ^{22}Na content of the pellets were measured, together with the specific activity of the supernatant. Sodium influx, J_{in}^{Na}, was calculated and expressed in μmol g^{-1} d.c.s min^{-1}.

In the experiments involving changes in cell volume, several protocols were employed. Cells were usually transferred directly from iso-osmotic to hyperosmotic salines containing Na^+, thus causing red cell shrinkage, followed by a progressive recovery to their initial volume resulting from the entry of Na^+ and Cl^- following activation of the Na^+/H^+ exchanger. In a second protocol, cells were shrunk in Na^+-free hyperosmotic medium. In this experimental condition, the Na^+/H^+ exchanger was activated, but the driving forces for net movement of ions were small and there was no discernible change in cell volume for 1 h. The activation of the Na^+/H^+ exchanger can be studied, however, by adding ^{22}Na at a tracer dose and measuring its uptake. In some experiment, cells that had been shrunk in a Na^+-free hyperosmotic medium were transferred to a Na^+-containing

hyperosmotic medium of identical osmolarity, and the time courses of J_{in}^{Na} and cell volume recovery were followed. Finally, in a third protocol, the time course of deactivation of the exchanger was followed by transferring the cells from a Na^+-free hyperosmotic medium to a Na^+-free iso-osmotic solution.

Measurements of intracellular pH

Cells were frozen rapidly, then thawed for 5 min and refrozen; the pH measurements were made immediately after a second thawing using a micro pH electrode.

Materials

The phosphatase inhibitors were okadaic acid from Novodirect (France) and calyculin A from Tebu (France). ^{22}Na was from Amersham Corporation. All other chemicals used were reagent grade.

Results

Comparative effects of kinase stimulation on trout and eel antiporters

Fig. 1A,B depicts the time courses of cell volume changes and unidirectional Na^+ influx (J_{in}^{Na}) for trout and eel erythrocytes after the addition of 5×10^{-7} mol l^{-1} isoprenaline in a nitrogen atmosphere. Addition of 10^{-3} mol l^{-1} 8-bromoadenosine 3′,5′-monophosphate (8Br-cyclic-AMP) gave identical results (data not shown). As previously shown (Baroin *et al.* 1984; Mahé *et al.* 1985), these compounds strongly stimulate trout red cell Na^+/H^+ exchangers *via* activation of the cyclic-AMP-dependent protein kinase A (PKA), which leads to considerable swelling of the erythrocytes as a result of a net uptake of NaCl *via* the Cl^-/HCO_3^- exchanger functionally coupled to the Na^+/H^+ exchanger. In contrast, the two compounds were found to be without significant effect on eel erythrocyte Na^+/H^+ exchangers.

Fig. 2A,B depicts the time courses of cell volume changes and unidirectional Na^+ influx for trout and eel erythrocytes after the addition of phorbol ester, an agonist of protein kinase C (PKC). As previously shown (Guizouarn *et al.* 1993), the trout red cell exchangers were activated by phorbol ester, even though the activation was less marked than that induced by β-agonists (note the difference in the scales in Figs 1 and 2). Clearly, eel erythrocyte Na^+/H^+ exchangers, unlike trout red

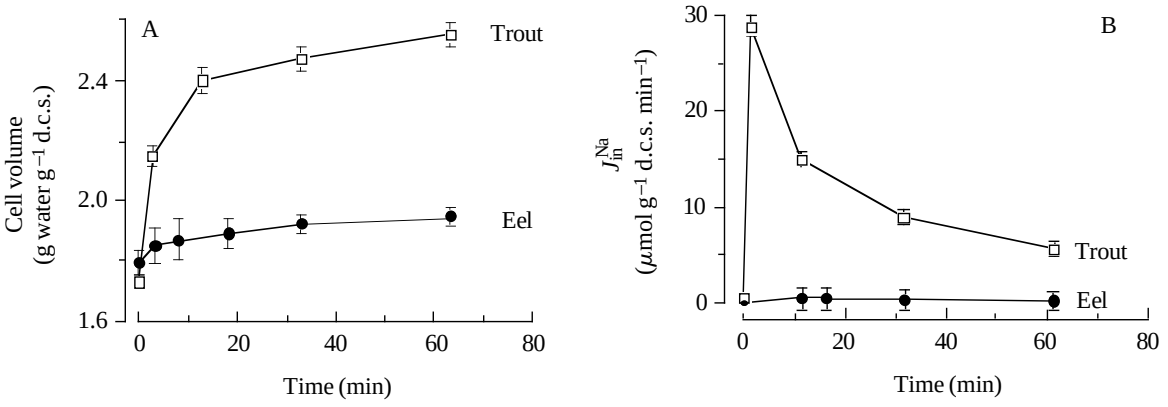

Fig. 1. Comparative effects of catecholamine on trout and eel erythrocytes. Cell volume (A) and Na^+ influx rate J_{in}^{Na} (expressing Na^+/H^+ exchange activity) (B) as functions of time after the addition of 0.5 μmol l^{-1} isoprenaline to erythrocytes suspended in an isotonic saline. ^{22}Na influx (3 min uptake) was measured in aliquots of each sample centred around the indicated times. Data for trout erythrocytes were obtained from previous experiments. Values are shown as means ± S.E.M., N=3 for eel, N=35 for trout. d.c.s., dry cell solids.

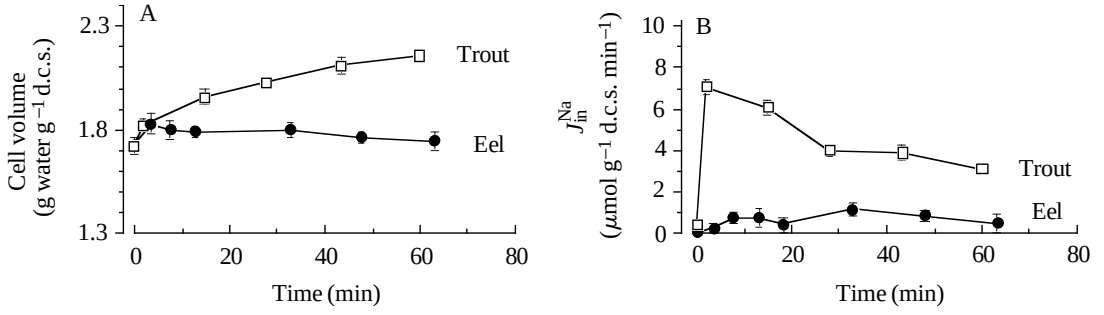

Fig. 2. Comparative effects of phorbol ester on trout and eel erythrocytes. Cell volume (A) and Na^+ influx rate J_{in}^{Na} (B) as functions of time after the addition of 1 μmol l^{-1} phorbol 12-myristate 13-acetate. Note that, in trout erythrocytes, activation of Na^+/H^+ exchange by phorbol ester is much less marked than activation by isoprenaline (see Fig. 1B). Values are shown as means ± S.E.M., N=3 for eel, N=10 for trout (where not indicated, the S.E.M. lies within the symbol).

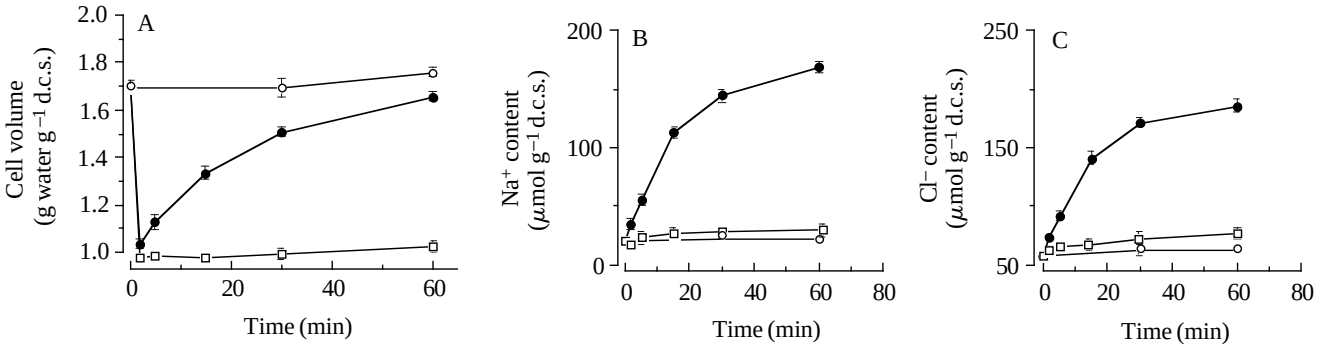

Fig. 3. Cell volume (A) and Na^+ (B) and Cl^- (C) contents as functions of time after osmotic shrinkage of eel erythrocytes in the absence (●) or presence (□) of the Na^+/H^+ exchange inhibitor amiloride (10^{-3} mol l^{-1}). Control erythrocytes (○) were maintained in isotonic saline. The osmolarity of the hypertonic medium was raised to 1.7 times that of control saline by the addition of NaCl. All media contained 1 mmol l^{-1} ouabain. Values are shown as means ± S.E.M., *N*=5.

cell antiporters, are not significantly activated by either PKA or PKC agonists.

Comparative effects of cell shrinkage

Fig. 3A depicts the time course of the change in cell volume of eel erythrocytes subjected to osmotic stress: cells were transferred to a solution with an osmolarity of 1.7 times the control osmolarity. This procedure resulted in an immediate osmotic shrinkage followed by a regulatory phase in which the volume returned to the control level in about 1 h. Amiloride completely inhibited volume recovery. This volume regulation is the result of a net uptake of NaCl caused by the functional coupling between Cl^-/HCO_3^- exchange and the amiloride-sensitive Na^+/H^+ exchange (Fig. 3B,C). In the presence of ouabain, after 60 min the net sodium uptake was 101.20±2.17 μmol g^{-1} d.c.s, the net chloride uptake 86.9±15.13 μmol g^{-1} d.c.s, and the net potassium uptake (not shown) was 5.90±5.69 μmol g^{-1} d.c.s (means ± S.E.M., *N*=7); the calculated volume increase due to NaCl entry was 0.52 g water g^{-1} d.c.s. compared with the observed volume increase of 0.48 g water g^{-1} d.c.s.

Fig. 4 compares the abilities of eel and trout erythrocytes to recover their initial volume after a hyperosmotically induced shrinkage of similar amplitude. In trout erythrocytes, volume recovery was only partial and the process stopped after about 1 h.

Characteristics of activation and deactivation of antiporters stimulated by cell shrinkage

Fig. 5A,B show the relationship between the Na^+/H^+ exchange rate and cell volume in eel erythrocytes. In Fig. 5A, the rate of unidirectional influx is plotted together with the corresponding cell volume as a function of time after cell shrinkage, the shrinkage being induced by transferring the cells from an iso-osmotic normal saline (Na^+-containing medium) to a hyperosmotic saline (obtained by the addition of 165 mosmol l^{-1} NaCl). Fig. 5B depicts a similar experiment but, in this case, the cells were transferred from an iso-osmotic normal saline to a nominally Na^+-free hyperosmotic medium (external sodium concentration 0.1 mmol l^{-1}). Under these experimental conditions, addition of ^{22}Na at a trace dose allows an analysis of the rate of Na^+/H^+ exchange as a function of time, but as significant Na^+ uptake cannot take place, cell volume recovery is prevented. Note that in both cases, although cell shrinkage was very rapid, the rate of Na^+ influx only increased gradually, taking about 10 min to reach a maximum value. Thus, activation of antiporters by cell shrinkage is a relatively slow process.

Figs 6B and 7 show that the magnitude of activation is a graded function of cell shrinkage. Fig. 6A depicts the time course of cell volume changes and Fig. 6B that of Na^+ influx when eel erythrocytes were osmotically shrunk by transfer from an iso-osmotic solution (220 mosmol l^{-1}) to different

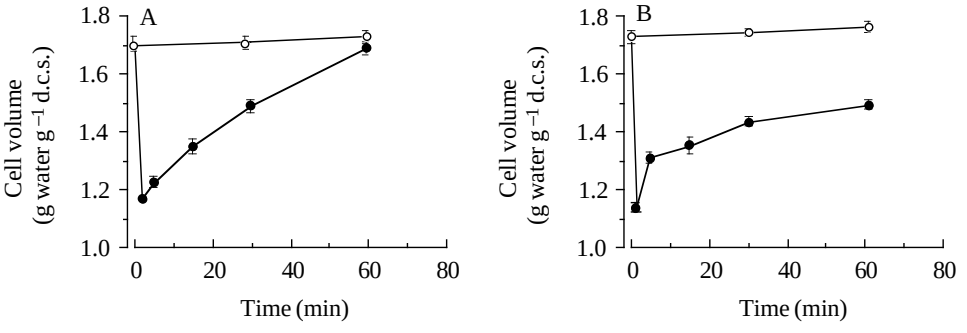

Fig. 4. Comparative capacities of eel (A) and trout (B) erythrocytes to regulate their volumes after osmotic shrinkage. Erythrocytes were exposed to hyperosmotic medium (●) or maintained in iso-osmotic saline (○). All media contained 1 mmol l^{-1} ouabain. Values are shown as means ± S.E.M., *N*=6.

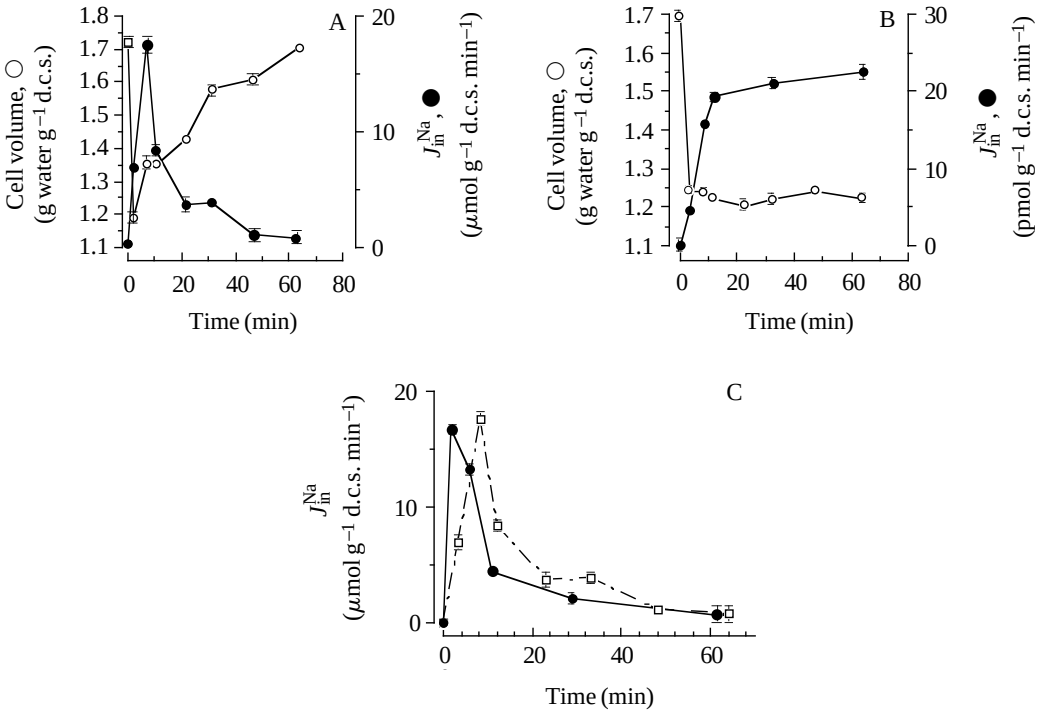

Fig. 5. Time course of Na^+/H^+ exchange (●) and cell volume (○) after transfer of eel erythrocytes from iso-osmotic saline to a Na^+-containing (A) or to a Na^+-free hyperosmotic (B) medium. The Na^+-containing and Na^+-free hyperosmotic media had the same osmolarity (1.6 times iso-osmotic). The rate of ^{22}Na influx and cell volume were simultaneously measured in small volumes of each sample. ^{22}Na influx was measured for 3 min around the indicated times. The nominally Na^+-free medium contained 0.1 mmol l^{-1} Na^+ and ^{22}Na at a trace dose. Note that incubation in Na^+-free hyperosmotic medium activates Na^+/H^+ exchange, but that the driving forces for net movement of ions under these circumstances are small and there is no discernible recovery of cell volume. (C) Time course of Na^+/H^+ exchange rate after transfer of eel erythrocytes to Na^+-containing hyperosmotic media. In one group (□), the cells were transferred directly from iso-osmotic saline to the hyperosmotic medium (replot of the data presented in A). The maximum rate of Na^+ influx (18 μmol g^{-1} d.c.s. min^{-1}; d.c.s., dry cell solids) was reached only after 10 min. In the second group (●), the cells were shrunk by incubation for 40 min in a hyperosmotic Na^+-free medium before being transferred to a Na^+-containing hyperosmotic medium of the same osmolarity. Under these conditions, there was no time lag, but the maximum Na^+ influx was also 18 μmol g^{-1} d.c.s. min^{-1}. Values are shown as means ± S.E.M. (where not indicated, the S.E.M. lies within the symbol), *N*=3.

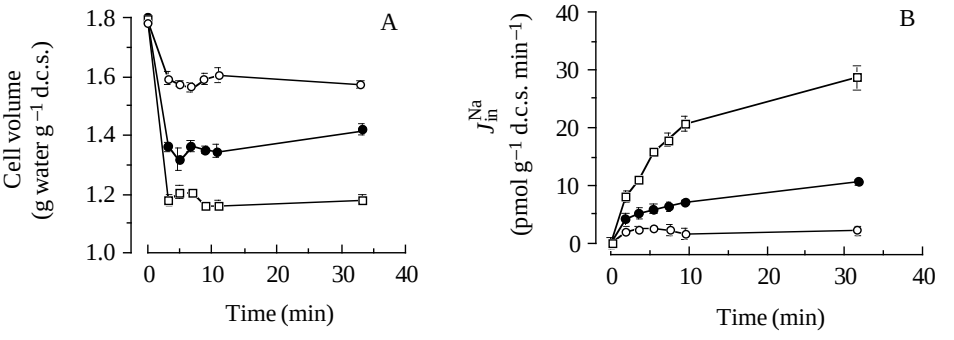

Fig. 6. Cell volume (A) and Na^+/H^+ exchange activity (B) as functions of time after transfer of eel erythrocytes to Na^+-free hyperosmotic media of different osmolarities. Cells were exposed to hyperosmotic media of 260 mosmol l^{-1} (○), 320 mosmol l^{-1} (●) and 380 mosmol l^{-1} (□). The different osmolarities were obtained by varying choline chloride concentration in the incubating medium. These results demonstrate that the rate of Na^+/H^+ exchange depends upon the degree of cell shrinkage. Values are shown as means ± S.E.M. (where not indicated, the S.E.M. lies within the symbol), *N*=3.

hyperosmotic Na^+-free media (+40 mosmol l^{-1}, +100 mosmol l^{-1}, +160 mosmol l^{-1}) in which the cells cannot recover their initial volumes after shrinkage. Fig. 7 is a plot of Na^+ influx as a function of cell volume: the cells were transferred from an iso-osmotic solution to different hyperosmotic media containing 85 mmol l^{-1} Na^+, and the Na^+ influxes measured 5–10 min later were plotted as a function of the cell volume measured immediately after shrinkage. All these data show that the degree of activation of antiporters is finely adjusted to the degree of cell shrinkage: the greater the shrinkage, the greater the activation.

Figs 5 and 6 also show that the antiporters, once activated

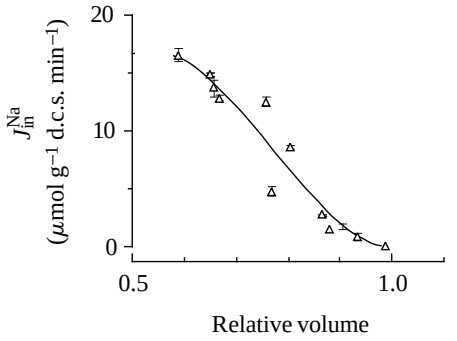

Fig. 7. Rate of Na^+/H^+ (J_{in}^{Na}) exchange activity as a function of the relative volume of eel erythrocytes. ^{22}Na influx was measured in media (85 mmol l^{-1} Na^+) of varying osmolarities produced by the addition of different concentrations of choline chloride. The abscissa represents the relative volume of shrunken cells, the reference being the volume of cells suspended in an iso-osmotic saline. 1 mmol l^{-1} ouabain was added to all media. Values are shown as means ± S.E.M. (where not indicated, the S.E.M. lies within the symbol), *N*=3.

by shrinkage, can be rapidly deactivated, or not, depending on the ability of the cells to recover their initial volume. When the cell recovered its volume (Fig. 5A) the rate of Na^+ influx, maximal at 10 min, decreased rapidly in an exponential manner to reach a value close to the basal value after 1 h; thus, deactivation started when only 30 % of cell volume had been regained and full deactivation occurred after complete recovery. Conversely, the experiments performed in Na^+-free media (Figs 5B, 6) show that antiporters remain activated as long as the cells remain shrunk irrespective of the level of activation imposed by the degree of cell shrinkage. This explains why, as illustrated in Fig. 5C, when erythrocytes have been preincubated in a Na^+-free hyperosmotic medium for 40 min before being transferred to a Na^+-containing medium with the same osmolarity, the rate of Na^+ influx does not increase progressively but is immediately maximal (the dashed line in Fig. 5C reproduces the results from erythrocytes not preincubated in a Na^+-free medium shown in Fig. 5A).

Fig. 8 indicates clearly that the degree of cell shrinkage controls the deactivation of antiporters. In this experiment, two batches of erythrocytes were transferred from an iso-osmotic saline to a hyperosmotic one; after shrinkage, cells started to recover their initial volume (Fig. 8A) by increasing the rate of Na^+ influx (Fig. 8B). After 18 min, successive additions of sucrose were made to one of the two batches to counteract cell volume recovery and to maintain these cells at a constant volume (Fig. 8A). Under these conditions, the rate of Na^+ influx remained high and constant (Fig. 8B), despite the fact that antiporters were causing a net Na^+ uptake. In conclusion, as long as cells remain in a shrunken conditions, antiporters remain activated, whether they transport ions or not, and deactivation is a graded and inverse function of the volume of shrunken cells; it starts when 30 % of the cell volume has been regained and is complete when the initial volume is reached.

The data in Fig. 9 show the time required for antiporter deactivation when the volume recovery is practically instantaneous. In this experiment, red cells were shrunk in a Na^+-free hyperosmotic medium for 30 min (conditions in which antiporters are maximally activated but cells cannot regulate their volume) and subsequently transferred back to an iso-osmotic Na^+-free saline; this transfer results in a very fast return to the normal cell volume by osmotic entry of water. Note that full deactivation occurred only about 20 min later. Subsequent hypertonic shock results in reactivation.

The pattern of activation of trout Na^+/H^+ antiporters by cell shrinkage is very similar to that described for eel antiporters, i.e. slow and progressive activation (taking about 15 min); also, the antiporters did not deactivate in the absence of cell volume recovery in Na^+-free medium (data not shown). However, there are two differences between the trout and eel antiporters: for a similar degree of cell shrinkage, the rate of Na^+/H^+ exchange is significantly lower in trout red cells and, in addition, trout antiporters become fully deactivated well before the cell volume has returned to its initial value, resulting in an incomplete volume recovery (see Fig. 11).

Deactivation versus *desensitization*

Fig. 10 shows that eel erythrocyte antiporters, activated by an osmotic shrinkage and then deactivated 1 h later after the cell volume had recovered, can again be fully reactivated by a second osmotic stress and initiate a new cell volume recovery. Thus, when cells start to regain their initial volume, the Na^+/H^+ exchange activity decreases progressively and finally stops, but

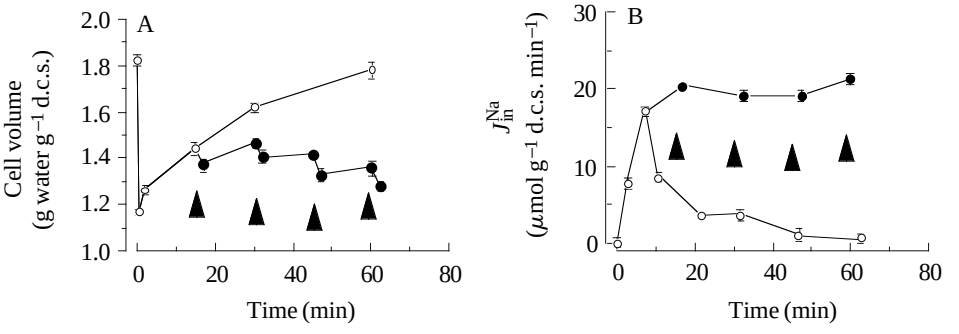

Fig. 8. Cell volume (A) and Na^+/H^+ exchange activity (B) as functions of time after osmotic shrinkage of eel erythrocytes either kept shrunk (●) or allowed to recover to their initial volume (○). Successive additions of 42 mosmol l^{-1} sucrose (arrows) prevented cell volume recovery. Note that, when red cells were maintained in a shrunken condition, Na^+/H^+ exchange activity remained constant and at its maximal rate. Values are shown as means ± S.E.M. (where not indicated, the S.E.M. lies within the symbol), *N*=3.

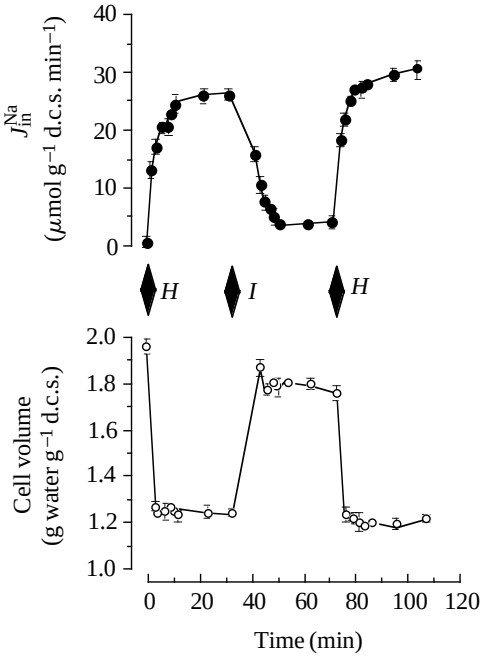

Fig. 9. Time course of activation and deactivation of the shrinkage-induced Na^+/H^+ exchanger in eel erythrocytes. Activation of Na^+/H^+ exchange was induced by transferring red cells to a Na^+-free hyperosmotic medium (*H*). After a time lag, the exchange rate (●) reached a maximum value at 10 min and remained constant, no cell volume (○) recovery occurring in the Na^+-free medium. The initial volume was then rapidly restored by transferring the cells at 30 min to an iso-osmotic Na^+-free medium (*I*). The Na^+/H^+ exchanger deactivated progressively but reached full deactivation only after 20 min in the isotonic solution. At this time, a new hypertonic shock induces an influx of Na^+ with the same time course as the first one. Values are shown as means ± S.E.M. (where not indicated, the S.E.M. lies within the symbol), *N*=3.

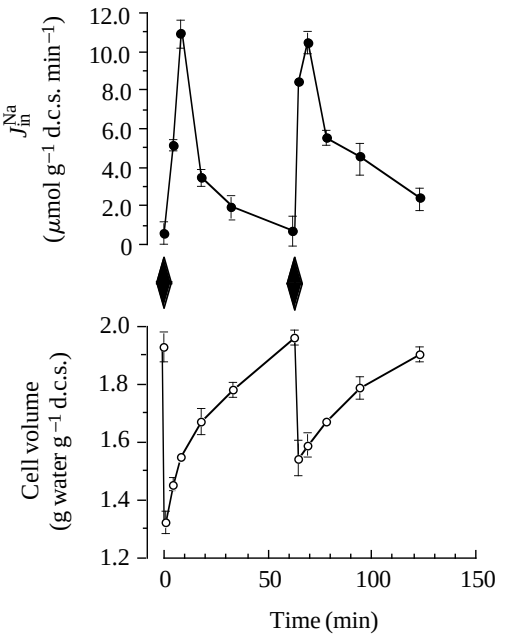

Fig. 10. The effect of two successive hyperosmotic stresses (arrowheads) on Na^+/H^+ exchange rate (●) and volume (○) of eel erythrocytes. Red cells were first transferred to a Na^+-containing hyperosmotic solution. After 60 min, when the cell volume had recovered and Na^+/H^+ exchangers were deactivated, a second hyperosmotic stress was imposed by the addition of NaCl (final osmolarity 324 mosmol l^{-1}). Note that Na^+/H^+ exchangers were fully reactivated by the second hyperosmotic stress. Values are shown as means ± S.E.M., *N*=3.

the antiporters can at any time immediately respond to a new osmotic challenge. Such a 'deactivation' of the antiporter is unrelated to the antiporter 'desensitization' observed when trout red cells are stimulated by catecholamine, cyclic AMP or forskolin. It has already been shown that exposure of trout erythrocytes to isoprenaline or cyclic AMP induces a rapid and considerable activation followed by a progressive decrease in exchange activity (see Fig. 1B). After removal of the agonist by washing the cells for 1 h after hormonal stimulation, the antiporters show a reduced ability to respond to a fresh challenge of hormone or cyclic AMP (Garcia-Romeu *et al.* 1988). In other words, in this case, the decline of activity does not reflect a simple activation–deactivation transition, but the shift of antiporters from an active to a refractory non-responsive state. This process has been termed desensitization (Garcia-Romeu *et al.* 1988).

We investigated whether the decrease in the exchange activity observed after activation by shrinkage of trout erythrocytes is a result of deactivation (as in eel erythrocytes) or desensitization (as when these cells are hormonally stimulated). Fig. 11 illustrates the results of an experiment similar to that described in Fig. 10. It is clear that trout erythrocytes respond immediately and fully to the second osmotic challenge, as do eel erythrocytes. In conclusion, the trout antiporter βNHE, which is desensitized after hormonal activation, is deactivated and not desensitized after osmotic activation.

Effect of phosphatase inhibitors

In *Amphiuma* erythrocytes, volume regulation following cell swelling is due to activation of a K^+/H^+ exchanger which mediates net K^+ loss. This type of volume regulatory mechanism is unique, and it has been suggested that these K^+/H^+ exchanges may be mediated by the Na^+/H^+ exchanger operating in a different mode. In view of this, we decided to look for the existence of K^+/H^+ exchanges in eel erythrocytes but we failed to observe such a process after osmotically induced swelling (data not shown).

Although in various cell types, including trout erythrocytes (Cossins *et al.* 1994), K^+ loss responsible for a regulatory volume decrease (RVD) appears to be activated by dephosphorylation, in *Amphiuma* red cells K^+/H^+ exchange activation is brought about by phosphorylation-dependent events. Exposing red cells in an iso-osmotic medium to the phosphatase inhibitor calyculin A resulted in a net K^+ loss (mediated by K^+/H^+ exchanges) and a Na^+ gain (mediated by

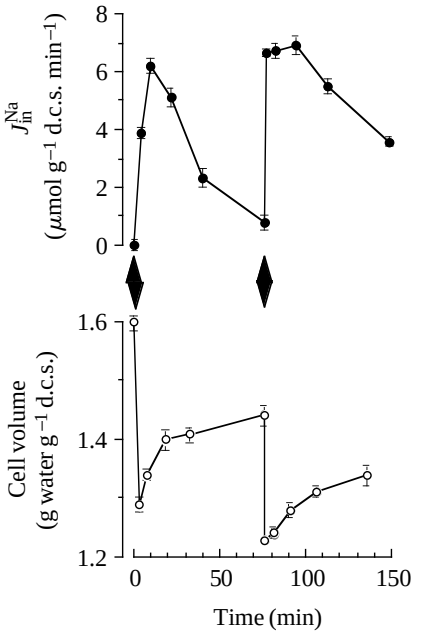

Fig. 11. Effect of two successive hyperosmotic stresses (arrowheads) on Na^+/H^+ exchange rate (●) and volume (○) of trout erythrocytes. Experimental conditions as in Fig. 10. Note that trout Na^+/H^+ exchangers were fully reactivated by the second osmotic stress. Values are shown as means ± S.E.M., *N*=4.

Na^+/H^+ exchanges) at nearly identical rates (Cala *et al.* 1993). Fig. 12A shows that 0.2 μmol l^{-1} calyculin A added to eel erythrocytes suspended in an iso-osmotic saline results in the activation of a Na^+ influx which increases progressively for the first 10 min after addition of calyculin A and then decreases to zero after 1 h. This Na^+ influx is amiloride-sensitive and promotes external acidification when the anionic exchanger is blocked by DIDS (4,4′-diisothiocyanato-stilbene-2,2′-disulphonic acid; data not shown), indicating that calyculin A activates Na^+/H^+ exchange. This activation resulted in an increase in cell volume (Fig. 12B) due to the net uptake of Na^+ and Cl^- (Fig. 12D) *via* the parallel functioning of Na^+/H^+ and Cl^-/HCO_3^- exchanges. It should be noted that the cell K^+ content remained constant (Fig. 12C), indicating that calyculin A did not promote the activation of a putative K^+/H^+ exchange, but did prevent the K^+ loss that is normally induced by cell swelling.

Fig. 12A,B also shows the effects of calyculin A on osmotically shrunken cells. After transfer of erythrocytes to a

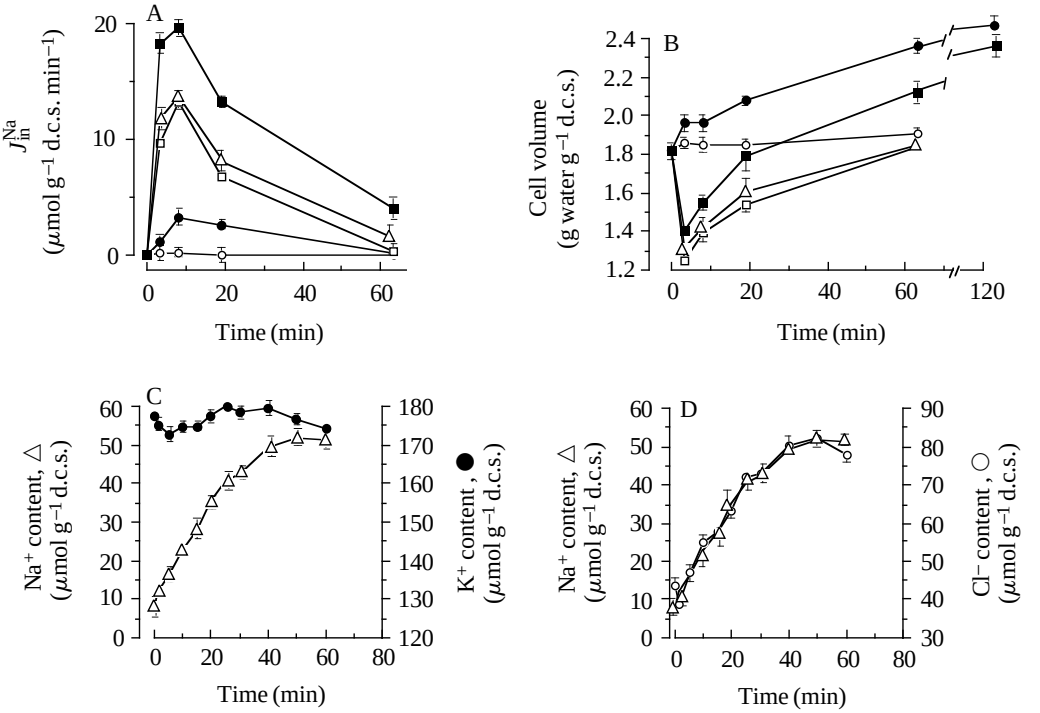

Fig. 12. Effects of phosphatase inhibitors on the Na^+/H^+ exchange rate (A), cell volume (B) and Na^+, K^+ and Cl^- contents (C,D) of normal and shrunken eel erythrocytes. (A,B) The phosphatase inhibitors were added either to eel red cells suspended in an iso-osmotic saline (normal cell volume) or to red cells exposed to a hyperosmotic stress (shrunken cells). Na^+/H^+ exchange rate (A) and cell volume (B) as functions of time for cells suspended in an iso-osmotic saline containing 5 μmol l^{-1} okadaic acid (○) or 0.2 μmol l^{-1} calyculin A (●); Na^+/H^+ exchange rate and cell volume following osmotic shrinkage in the absence of phosphatase inhibitors (control cells □), in the presence of okadaic acid (△) or calyculin A (■). Note that okadaic acid had no effect in either experimental condition. Conversely, in the presence of calyculin A, exchange was activated in normal cells, and shrunken cells regulated their volume more rapidly and reached a new steady state. (C,D) Effect of calyculin A on the Na^+ and K^+ (C) and Na^+ and Cl^- (D) contents of osmotically shrunken cells. Note that calyculin A did not induce K^+ loss and resulted in identical Na^+ and Cl^- gains. Values are shown as means ± S.E.M., *N*=3.

hyperosmotic saline, the patterns of Na^+ influx with or without calyculin A were similar, but the magnitude of the flux was consistently higher in the presence of calyculin A (Fig. 12A). As a consequence, shrunken cells treated with calyculin A recovered their initial volume more rapidly than control cells (20–30 min and 60 min, respectively) and continued to swell (Fig. 12B).

It should be noted that another phosphatase inhibitor, okadaic acid, had no significant effect on Na^+ influx and cell volume either in iso-osmotic saline or in osmotically shrunken cells (Fig. 12A,B).

Discussion

The regulatory properties of Na^+/H^+ antiporters of nucleated erythrocytes

The trout and eel red blood cell Na^+/H^+ exchangers have been shown to have widely different regulatory properties. Hyde and Perry (1990) demonstrated the adrenergic insensitivity of American eel (*Anguilla rostrata*) red blood cells *in vivo* when the eel is exposed to hypoxia or acidosis, conditions known to enhance the catecholamine-mediated erythrocyte response in trout. We confirmed *in vitro*, using the European eel (*Anguilla anguilla*), that catecholamine and cyclic AMP do not produce significant activation of the red cell Na^+/H^+ exchangers (Fig. 1). Similarly, phorbol esters, which activate the trout red cell exchanger βNHE, do not affect the eel exchanger. Furthermore, eel red blood cells show a remarkable capacity for volume regulation: after exposure to 1.7-fold hyperosmotic saline, the initial cell volume recovers fully in 1 h. This volume-induced process, which is faster even than that of *Amphiuma* erythrocytes, involves the activation of an amiloride-sensitive Na^+/H^+ exchanger that is functionally coupled to the anion exchanger. In contrast, trout red cells exposed to similar variations of osmotic pressure tend to regulate their volume by activating Na^+/H^+ exchange, but activation is only partial since they recover less than half their volume (Fig. 4).

Thus, the regulatory properties of the eel red cell antiporter are very different from those of the trout red cell exchanger but are similar to those described by Cala (1983) for the *Amphiuma* red cell exchanger. These different regulatory properties probably indicate the existence in nucleated erythrocytes of different isoforms of the exchanger, a hypothesis essentially based on the observation that the sensitivity of βNHE to catecholamines is strictly related to the presence of PKA consensus sites located on the cytoplasmic domain of the antiporter: the point mutation of serine in these sites inhibits catecholamine-induced activation, and grafting these sites to the human exchanger NHE1, normally insensitive to catecholamine, confers a catecholamine-dependence to NHE1 (Borgese *et al.* 1994).

Characteristics of shrinkage-activated Na^+/H^+ exchange

Our experiments have demonstrated that eel erythrocytes, after osmotic shrinkage, show a remarkable capacity to return to the volume they had in an isotonic medium. This regulatory volume increase (RVI) is mediated by the activation of Na^+/H^+ exchangers; RVI stops when the initial volume is attained, indicating that all the antiporters are turned off. The term 'volume set point' is frequently used to define the volume at which all volume-sensitive transporters are inactive, any deviation from this set point resulting in the activation of shrinkage-activated or swelling-activated pathways.

In a series of careful studies, Cala showed that the shrinkage-activated *Amphiuma* exchangers possess regulatory properties very similar to those described in this paper for the shrinkage-activated eel exchanger (Cala, 1986; Maldonado and Cala, 1992). The main characteristics of eel antiporter activation and deactivation following exposure to a hyperosmotic medium are listed below.

Activation of the Na^+/H^+ exchanger is induced by cell shrinkage and occurs even in the absence of Na^+ in the hypertonic medium (Fig. 5A,B), but the magnitude of Na^+/H^+ exchange activation is a graded function of cell shrinkage. The activation is not instantaneous: after shrinkage, both in Na^+-containing and in Na^+-free medium, the Na^+/H^+ exchange rate gradually increases, reaching a maximum value after about 10 min (Fig. 5A,B). This time lag between osmotic shrinkage and Na^+/H^+ exchange activation is roughly the same irrespective of the degree of osmotic shrinkage (Fig. 6).

Deactivation, like activation, is induced by volume change: as cells progressively swell during RVI, the rate of Na^+/H^+ exchange decreases simultaneously (Fig. 5A). If cells are kept shrunken either by successive small additions of hypertonic medium to cancel RVI (Fig. 8) or by shrinking cells in a Na^+-free medium in which no net ion uptake is possible (Fig. 5B), their antiporters remain activated and the rate of Na^+/H^+ exchange is maintained constant. Like activation, deactivation also occurs after some delay, as can be seen in Fig. 9. Here, cells shrunk in a Na^+-free hypertonic medium for 30 min were resuspended in a Na^+-free isotonic medium in which their volume was rapidly restored. The Na^+/H^+ exchange rate decreased, but deactivation was only complete 20 min later.

Volume change is clearly the signal for both activation and deactivation of antiporters. However, perception of the signal by cells and its transduction are complex phenomena, as illustrated by the following example. Fig. 5 shows that the magnitude of activation of Na^+/H^+ exchange is the same in erythrocytes shrunk by preincubation in a Na^+-free hypertonic medium and then transferred to a Na^+-containing hypertonic saline of the same osmolarity (no lag time for activation) as in erythrocytes transferred directly to the Na^+-containing hypertonic saline (progressive activation). This indicates that the magnitude of activation is strictly controlled by the initial degree of cell shrinkage, but is subsequently unaffected by the partial cell volume recovery that occurs during the progressive activation (about 10 min).

βNHE, the trout antiporter, is much less sensitive to cell shrinkage than the eel antiporter: for a shrinkage of similar magnitude, βNHE carries fewer ions. In addition, it is deactivated prior to restoration of the normal control volume,

leaving cell volume regulation notably defective (Fig. 11). It has been suggested that in human lymphocytes a similar failure of volume regulatory capacity could be due to pH-dependent, and not to volume-dependent, deactivation of antiporters (Grinstein *et al.* 1985). Whether this could explain the situation in βNHE is, as yet, unknown.

Role of phosphorylation in RVI and in the expression of a putative K^+/H^+exchange

A number of findings point to the involvement of phosphorylation–dephosphorylation reactions in the control of volume-activated transport systems. Work on red blood cells has led to the hypothesis that a protein kinase plays a pivotal role. Shrinking the cell stimulates a kinase, leading to activation of transporters involved in regulatory volume increase (RVI), such as the Na^+/H^+ exchanger and $Na^+/K^+/2Cl^-$ cotransport. Swelling inhibits the kinase, leading to activation of the transporters involved in regulatory volume decrease (RVD), such as K^+/Cl^- cotransport (Jennings and Al-Rohil, 1990; Parker *et al.* 1991; Cossins, 1991; Starke and Jennings, 1993). The target of the kinase may be the transporters. The two types of transporter, however, always appear to be regulated in a coordinated fashion: agents stimulating one type inhibit the other and *vice versa* (Parker *et al.* 1990), suggesting that a 'regulator' controls the two types of transport system in a reciprocal manner and orchestrates the responses of the cell (Parker, 1993). In this context, the regulator might also be a putative target of the kinase. Phosphatase inhibitors, by shifting the phosphorylation–dephosphorylation equilibrium towards phosphorylation, were shown to have major effects on volume-sensitive transport systems, i.e. stimulation of shrinkage-activated transporters such as the Na^+/H^+ exchanger and inhibition of swelling-activated transporters such as K^+/Cl^- cotransport (Jennings and Schulz, 1990; Bianchini *et al.* 1991; Parker *et al.* 1991; Cossins *et al.* 1994; Guizouarn *et al.* 1995).

The results obtained with *Amphiuma* red cells do not fit well into this general scheme. In these erythrocytes, the shrinkage-activated pathway is a Na^+/H^+ exchanger and the swelling-activated pathway a K^+/H^+exchanger. A K^+/H^+ exchanger as a volume-sensitive transport system has only been described in *Amphiuma* red cells (Cala, 1985). Cala (1986) also suggested that, in these cells, the Na^+/H^+ and K^+/H^+ exchangers could represent alternative forms of the same transport moiety, an interesting possibility which, to our knowledge, has never been considered in relation to other isoforms of Na^+/H^+ antiporters. As in other cells, the two pathways are normally regulated in a coordinated fashion, i.e. when one is turned on, the other is turned off. However, addition of calyculin A, a phosphatase inhibitor, to erythrocytes suspended in an isotonic medium activated Na^+/H^+ exchange but also, unexpectedly, swelling-activated K^+/H^+ exchange, resulting in net K^+ loss and Na^+ gain at nearly identical rates (Cala *et al.* 1993).

In view of similarities between the Na^+/H^+ antiporters of eel and *Amphiuma* erythrocytes, it was of interest to analyze the effects of phosphatase inhibitors on eel erythrocytes, especially in relation to the expression of a putative K^+/H^+ exchange.

Exposing eel red cells in iso-osmotic medium to calyculin A resulted in cell swelling due to the activation of the Na^+/H^+ exchanger, but the red cell K^+ content remained constant, indicating that the phosphatase inhibitor had not induced a K^+ loss (Fig. 12). Thus, the calyculin-A-dependent phosphorylation does not reveal a silent K^+/H^+ exchange in the eel erythrocyte, as in *Amphiuma* erythrocyte. Furthermore, we also failed to activate K^+/H^+ exchange by osmotically induced cell swelling (data not shown).

In the volume-activated mode, Na^+/H^+ antiporters do not desensitize

When eel and trout red cell Na^+/H^+ antiporters have been activated by cell shrinkage, their activity decreases progressively as a function of time, but they can immediately be restimulated by a new osmotic challenge (Figs 10, 11). Thus, after volume activation, the observed decline of activity reflects a progressive deactivation of the antiporters.

In contrast, the decline of activity observed after hormonal stimulation of βNHE, the trout antiporter (Fig. 1B), does not reflect such a simple activation–deactivation transition, but a transition from an active to a refractory state: βNHE can no longer be immediately reactivated by a fresh challenge with catecholamine or cyclic AMP, and several hours without stimulation are necessary for the exchanger to recover its ability to respond to cyclic AMP or catecholamine. This desensitization is controlled by a phosphorylation–dephosphorylation reaction and may be the result of withdrawal of the protein from the cell surface and its sequestration in an altered conformation (Guizouarn *et al.* 1993, 1995).

The same Na^+/H^+ exchanger βNHE, once activated, can be either desensitized or simply deactivated depending on the nature of the stimulus. The reason for such divergent behaviour is unknown, but could be related to whether the antiporter is, or is not, phosphorylated: there is quite convincing evidence that catecholamine-induced activation is associated with phosphorylation of βNHE, since the point mutation of only one serine of the PKA consensus sites located on the cytoplasmic domain of the exchanger drastically inhibits activation (Borgese *et al.* 1994). In contrast, shrinkage-induced activation of NHE1, the human antiporter, has been shown not to be associated with phosphorylation of the transport molecule (Grinstein *et al.* 1992). This finding does not invalidate the model in which the kinase stimulation caused by shrinkage is likely to be responsible for the Na^+/H^+ exchange. The substrate for the shrinkage-induced phosphorylation would not be the exchanger itself but another element, such as the regulator proposed by Parker (1993).

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